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The Role of Cuticular Hydrocarbons in Mating and Conspecific Recognition in the Closely Related Longicorn Beetles *Pidonia grallatrix* and *P. takechii*

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The role of cuticular chemicals in mating behavior and their chemical components were studied in two sympatric flower-visiting longicorn beetles, *Pidonia grallatrix* and *P. takechii*. Mating experiments revealed that female cuticular chemicals elicit male mating behavior and that males can discriminate between conspecific and heterospecific females on the basis of contact chemicals. GC-MS analyses of whole-body extracts in the two species and both sexes determined that extracts contained a series of hydrocarbons including *n*-alkanes, *n*-alkenes, and methylalkanes. The relative abundance of some hydrocarbons differed between species and sexes, and canonical discriminant analysis showed discrimination of species and sex could be made unambiguously with several compounds. These results imply that the difference in cuticular hydrocarbons facilitates the premating isolation of sympatric *Pidonia* species.

Key words: *Pidonia*, longicorn beetles, sex pheromones, cuticular hydrocarbons, mating behavior, coexistence

INTRODUCTION

Many studies have demonstrated that longicorn beetles use sex pheromones in mating behavior (Akutsu and Kuboki, 1983; Wang *et al*., 1991; Fukaya and Honda, 1992; Kim *et al*., 1993; Fukaya and Honda, 1995; Ginzel and Hanks, 2003; Ginzel *et al*., 2003b; Zhang *et al*., 2003; Crook *et al*., 2004). In particular, cuticular hydrocarbon constituents serve as contact sex pheromones in some cerambycid groups (Fukaya *et al*., 1996; Fukaya *et al*., 2000; Ginzel *et al*., 2003a, b; Zhang *et al*., 2003). For example, male mounting behavior in the longicorn beetle *Psacothea hilaris* is elicited by male antennal contact with the female (Fukaya and Honda, 1992). However, sex pheromones of longicorn beetles have been studied mainly in relation to the biology and control of pest insects (Allison *et al*., 2004). Phytophagous beetles, including longicorn beetles, have diversified in association with angiosperms (Farrell, 1998). Contact pheromones may play an important role in the divergence of phytophagous insects by serving as prezygotic isolation mechanisms.

Flower-visiting longicorn beetles of the genus *Pidonia* (Cerambycidae: subfamily Lepturinae) have undergone a marked radiation within the Japanese archipelago, where this genus consists of 56 species, of which 96% are endemic to Japan (Kuboki, 1987; Conversazione of *Pidonia* ed., 2003). Furthermore, a number of closely related species often coexist with large niche overlap. For example, in a mountain area of central Honshu, adults of as many as 16 species are active during the same time of year in a single valley, and adults of more than 10 species feed and mate on the same flowers (Kuboki, 1980). This implies that rigid mate-recognition mechanisms facilitate the coexistence of *Pidonia* species. However, mate-recognition cues and their differences between species have not been studied in *Pidonia* longicorn beetles.

The purpose of this study was to elucidate the role of cuticular hydrocarbons in mate recognition through mating experiments using two closely related sympatric species, *P. grallatrix* and *P. takechii*. In addition, cuticular hydrocarbons (CHC) were analyzed using gas chromatography–mass spectrometry (GC–MS), and the composition of CHC between sexes and species was compared.

MATERIALS AND METHODS

Insects

Two sympatric species of *Pidonia*, both belonging to the subgenus *Pidonia*, *P. (P.) grallatrix* and *P. (P.) takechii* (Coleoptera, Cerambycidae), were used for mating experiments and characterization of cuticular hydrocarbons. Adults of both species were captured by sweep net from flowers of two plant species, *Swida controversa* and *Filipendula kamtschatica*, in Ougisawa Valley, Nagano Prefecture, central Honshu, Japan in July 2002- and 2003. All individuals captured were brought to a field tent set up at Ougisawa and kept individually in plastic Petri dishes, fed with

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sugar water under natural light/dark and temperature conditions, and used in mating experiments one day after capture.

Mating experiments

A series of bioassays were conducted to confirm that males of *Pidonia* use contact pheromones to recognize conspecific females. The experiments were conducted in the field tent from 06:00–11:00 at approximately 22°C. For all experiments, 5-cm diameter plastic Petri dishes with covers were used, in which two males and a female were placed to observe mating behavior. Each individual was used only once to avoid contamination of cuticular chemicals. Two males were used in each experiment to increase encounter the frequency between the sexes. All individuals were allowed to move and have access to each other in the dish. Beetle behavior was observed for 10 min, and mounting and abdominal bending behavior of males against females, an indication of mate recognition (see Results), were recorded. If both behaviors were observed, we regarded mounting as having occurred.

Six different sets of mating experiments, with 30 replicates, were conducted:

1. A living conspecific female was presented to the males to study the repertoire of male mating behavior and to confirm their mating activity.
2. A living heterospecific female was presented to the males to confirm their ability to discriminate heterospecific females.
3. A female anesthetized with CO_2 was presented to the males to examine the role of female movement in mate recognition.
4. Females were immersed in 0.5 ml hexane five times just before the experiment to strip cuticular chemicals from the female body. After drying for 20 min, a solvent-washed (thus dead) female was presented to the males to examine the effect of female cuticular chemicals in eliciting male mating behavior.
5. A female in which cuticular chemicals were stripped and which was then coated with conspecific female extracts was presented to the males to examine the effect of female cuticular chemicals in eliciting male mating behavior. Prior to the experiment, an extract of female cuticular chemicals was prepared by immersing a female in 0.5 ml hexane for 5 min. The extract was chromatographed through 0.5 g hexane-filled silica gel (C-300HG; Wako Pure Chemical Industries, Osaka, Japan) to remove polar chemicals. All extracts were mixed and used for the experiments. Females used in the mating experiment were coated with the hexane extract, and the solvent was allowed to evaporate. Prior to the experiment, it was confirmed that coating a female with an 0.5-ml aliquot of hexane extract (*i.e*., 1 female equivalent) was sufficient to elicit male mating behavior. In addition, it was also confirmed that crude and chromatographed extracts do not differ in eliciting male mating activity and behavior.
6. A solvent-washed, heterospecific dead female coated with conspecific female extract was presented to the males to examine the interaction effects of the female body (heterospecific) and cuticular chemicals (conspecific). Preparations of the female extract and the coating method were as in treatment 5.

The difference in mounting rate between species or treatments was examined by Fisher's exact probability test.

Identification of cuticular chemicals

To identify the cuticular chemicals, these were extracted with hexane from each of five individuals for each sex and species by immersing an individual in a 1-ml aliquot of hexane for 5 min. Individual samples were concentrated to dryness under pure nitrogen and dissolved in hexane to a volume of 30 μl, and 1 μl was analyzed with GC–MS in the electron impact (EI) mode using a Shimadzu GCMS-QP5000 or QP5050A apparatus (Shimadzu Corp., Kyoto, Japan), each containing a DB-1 capillary column (30 m long, 0.25 mm inner diameter). The carrier gas was helium, and the oven temperature was programmed to ramp from 80 to 200°C at 20°C/min and from 200 to 300°C at 10°C/min. Total time of the program was 22 min. Prior to the above analysis, longer-time analyses were performed to confirm that compounds with longer chains were negligible. Injector and detector temperatures were maintained at 320°C. Injections were performed in splitless mode (60 sec). Alkanes and alkenes were identified by retention time and relative position of equivalent chain length (ECL) compared to standards of icosane, docosane, tetracosane, hexacosane, octacosane, and triacontane. Double-bond positions were determined after alkylthiolation (DMDS) (Scribe *et al*., 1988).

Statistical analysis

Each peak area in the GC–MS data was transformed by the following formula (Aitchison 1986): $Z=\ln[A_p/g(A_p)]$, where A_p is the area of the peak (with 1 added to avoid zero values) and $g(A_p)$ is the geometric mean of all peaks. The variation in Z-value of each hydrocarbon component between sex and species was analyzed by two-way ANOVA. In addition, canonical discriminant analysis was performed to find hydrocarbon components useful for discriminating species and sex. In this analysis, 35 of 70 hydrocarbons with mean relative abundance exceeding 1% in any species or sex were additionally entered into the model, unless the probability of the F value was<0.05 (forward-stepwise selection). Hydrocarbon components with a mean relative abundance of<1% were not distinguishable from noise and were not included in the analysis. These statistical analyses used JMP software (SAS Institute, 2002).

RESULTS

Mating behavior

A series of mating experiments in petri dishes allowed observation of the mating behavior of *Pidonia* beetles. The repertoire of male mating behavior did not differ between species. A male walking around in the petri dish detected a female when its antenna touched a part of a female body.

Table 1. Percentages of males of *P. grallatrix* and *P. takechii* mounting differently treated females of the two species.

	Response of *P. grallatrix* males		Response of *P. takechii males*	
Treatment*	Female	Mounting (%)	Female	Mounting (%)
1. Conspecific: intact	*P. grallatrix*	97	*P. takechii*	93
2. Heterospecific: intact	*P. takechii*	0	*P. grallatrix*	0
3. Anesthetized with CO_2	*P. grallatrix*	80	*P. takechii*	90
4. Washed by hexane	*P. grallatrix*	0	*P. takechii*	0
5. Conspecific body coated with conspecific extracts	*P. grallatrix* body + *P. grallatrix* extract	70	*P. takechii* body + *P. takechii* extract	70
6. Heterospecific body coated with conspecific extracts	*P. takechii* body + *P. grallatrix* extract	33	*P. grallatrix* body + *P. takechii* extract	70

* See text for details. For each treatment, 30 replicates were performed.

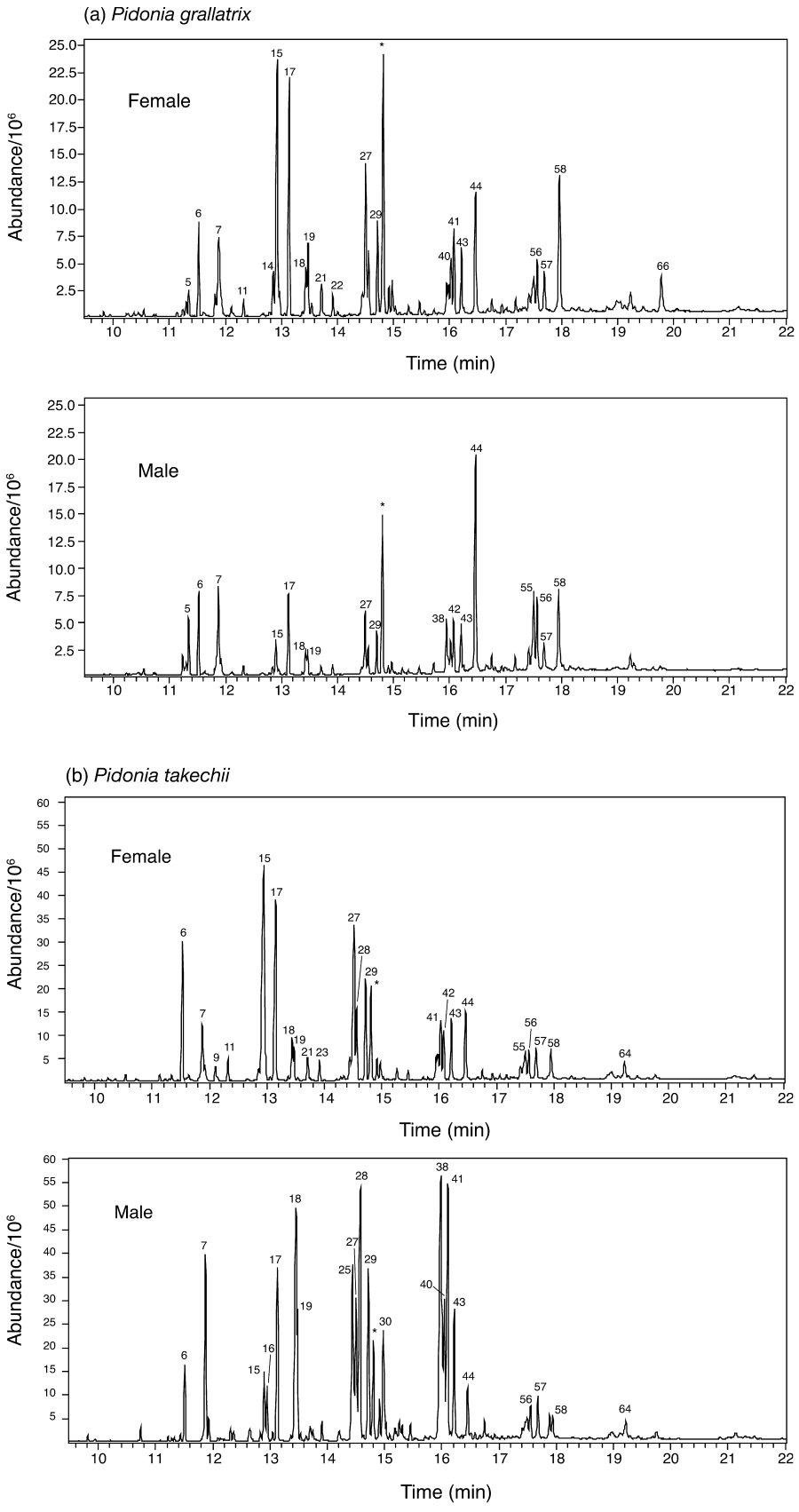

Fig. 1. Representative gas chromatograms of hexane extracts of **(a)** *P. grallatrix* and **(b)** *P. takechii* (top, female; bottom, male). Major peaks are labeled with the peak numbers given in Table 2. Star indicates phthalic acid ester from an inner lid of a vial.

Table 2. Cuticular hydrocarbon components of each sex of the two *Pidonia* species

			Pidonia grallatrix		*Pidonia takechii*			F^c		
Peak[a]	Hydrocarbon	ECL	Female	Male	Female	Male	Diagnostic ions	Species	Sex	Species × Sex
1	n-C_{20}	20.00	0.06±0.03[b]	0.08±0.03	0.09±0.02	0.20±0.21	282 (M^+)	0.34	1.30	2.53
2	C_{21}diene	20.59	0.19±0.11	1.00±0.14	0.07±0.06	nd	292 (M^+)	21.70***	17.52***	11.68**
3	4-MeC_{20}	20.64	nd	nd	nd	0.11±0.11	296 (M^+)	11.81**	14.61**	15.16**
4	(*Z*)-9-$C_{21:1}$	20.68	0.74±0.66	0.58±0.34	0.08±0.02	0.13±0.10	294 (M^+)	28.07***	0.74	0.00
5	(*Z*)-7-$C_{21:1}$	20.73	0.91±0.18	2.73±1.02	0.17±0.04	0.11±0.11	294 (M^+)	13.98**	2.23	4.81*
6	n-C_{21}	21.00	4.02±1.91	3.72±0.66	8.44±1.65	1.69±1.04	296 (M^+)	8.68**	13.25**	11.38**
7	7-MeC_{21}	21.40	2.94±1.38	4.73±1.39	2.21±1.09	4.92±3.58	112, 224, 295, 310 (M^+)	10.55**	0.04	0.73
8	5-MeC_{21}	21.49	0.42±0.33	0.60±0.44	0.24±0.19	0.96±0.97	84, 252, 295, 310 (M^+)	0.06	2.32	1.23
9	3-MeC_{21}	21.70	nd	0.22±0.09	0.49±0.40	nd	280, 295	0.39	0.39	452.62***
10	(*Z*)-9-$C_{22:1}$	21.72	0.26±0.13	nd	nd	nd	308 (M^+)	43.78***	37.31***	34.35***
11	n-C_{22}	22.00	0.73±0.28	0.73±0.60	0.94±0.32	0.47±0.20	310 (M^+)	5.70*	3.84	2.35
12	8-MeC_{22}	22.42	nd	nd	nd	0.50±0.30	126, 224, 309, 324 (M^+)	118.04***	137.28***	141.02***
13	4-MeC_{22}	22.64	0.27±0.28	0.55±0.21	nd	0.34±0.22	280, 309, 324 (M^+)	13.32**	37.12***	5.75*
14	C_{23}diene	22.67	1.13±1.03	nd	1.02±0.97	nd	320 (M^+)	1.42	51.89***	2.62
15	(*Z*)-9-$C_{23:1}$	22.75	9.18±6.74	3.02±0.63	10.68±10.23	2.14±0.89	322 (M^+)	6.19*	7.53*	3.97
16	(*Z*)-7-$C_{23:1}$	22.80	0.94±0.69	0.35±0.13	0.29±0.21	1.48±0.59	322 (M^+)	3.63	0.00	5.22*
17	n-C_{23}	23.00	9.73±2.50	5.81±1.29	10.37±0.67	8.20±4.11	324 (M^+)	4.33	4.55*	0.31
18	9-MeC_{23}	23.40	2.42±0.27	1.78±0.52	1.89±0.50	7.57±3.72	140, 224, 323, 338 (M^+)	1.60	0.69	2.47
19	7-MeC_{23}	23.44	1.61±0.86	1.21±0.31	1.00±0.35	2.03±1.07	112, 252, 323, 338 (M^+)	17.52***	0.01	0.06
20	5-MeC_{23}	23.53	0.26±0.23	nd	0.16±0.04	0.24±0.25	84, 280, 323, 338 (M^+)	14.25**	21.87***	8.29*
21	3-MeC_{23}	23.74	1.33±0.36	0.57±0.39	1.72±0.47	1.37±1.44	308, 323, 338 (M^+)	0.44	7.50*	0.79
22	(*Z*)-9-$C_{24:1}$	23.80	nd	nd	nd	0.25±0.18	336 (M_+)	12.65**	15.36**	15.89**
23	n-C_{24}	24.00	0.92±0.30	1.55±2.17	1.40±0.98	0.77±0.62	338 (M^+)	2.19	1.75	1.87
24	10-MeC_{24} 9-MeC_{24}	24.39	nd	nd	nd	0.25±0.16	154, 224, 337 140, 238, 337	12.66**	15.37**	15.91**
25	4-MeC_{24}	24.68	nd	nd	nd	4.20±3.95	308, 337	139.38***	158.64***	162.37***
26	C_{25}diene	24.68	1.96±1.57	0.84±0.20	1.61±1.04	nd	348 (M^+)	250.51***	224.66***	205.34***
27	(*Z*)-9-$C_{25:1}$	24.76	9.43±3.76	4.89±2.49	20.39±12.08	5.66±.06	350 (M^+)	1.48	14.40**	7.26*
28	(*Z*)-7-$C_{25:1}$	24.82	3.06±0.77	1.98±0.43	2.19±1.26	7.91±3.26	350 (M^+)	1.60	0.62	0.48
29	n-C_{25}	25.00	4.62±2.06	3.45±2.18	5.95±1.11	8.48±5.99	352 (M^+)	0.60	1.03	0.04
30	13-MeC_{25} 11-MeC_{25}	25.37	1.71±0.74	0.87±0.56	1.04±0.61	2.68±1.67	196, 351, 366 (M^+) 168, 224, 351, 366 (M^+)	0.32	1.11	1.16
31	7-MeC_{25}	25.44	0.18±0.10	0.45±0.74	0.12±0.03	0.19±0.14	112, 280, 351, 366 (M^+)	0.00	1.41	1.64
32	5-MeC_{25}	25.53	nd	nd	0.19±0.20	0.18±0.09	84, 308, 351	27.69***	0.02	0.01
33	C_{26}diene	25.69	nd	nd	nd	0.12±0.11	362 (M^+)	3.88	5.12*	5.36*
34	3-MeC_{25}	25.76	0.56±0.38	0.20±0.05	0.68±0.54	0.58±0.27	336, 366 (M^+)	1.16	0.13	0.78
35	(*Z*)-7-$C_{26:1}$	25.82	nd	nd	nd	0.25±0.15	364 (M^+)	12.68**	15.39**	15.92**
36	n-C_{26}	26.00	0.45±0.06	1.29±2.01	0.98±0.81	1.57±1.87	366 (M^+)	0.36	0.01	0.69
37	13-MeC_{26} 12-MeC_{26}	26.35	0.27±0.10	0.76±0.24	0.09±0.08	nd	196, 210, 365, 380 (M^+) 182, 224	28.00***	1.76	9.49**
38	4-MeC_{26}	26.65	0.69±0.80	4.17±1.78	1.00±0.60	6.04±5.01	336, 380 (M^+)	0.14	4.91*	0.61
39	C_{27}diene1	26.71	0.96±0.43	1.11±0.33	nd	nd	376 (M^+)	39.39***	36.67***	73.02***
40	C_{27}diene2	26.76	4.20±2.40	nd	1.60±0.77	3.87±2.01	376 (M^+)	0.15	0.63	149.91***
41	(*Z*)-9-$C_{27:1}$	26.83	5.45±4.39	1.94±0.48	3.78±1.07	5.31±3.32	378 (M^+)	3.22	4.20	0.07
42	(*Z*)-7-$C_{27:1}$	26.93	1.47±2.06	3.48±0.59	1.81±0.60	1.81±2.14	378 (M^+)	0.97	0.32	6.21*
43	n-C_{27}	27.00	2.60±0.07	3.59±1.03	3.56±0.41	5.39±4.45	380 (M_+)	2.86	0.04	1.31
44	13-MeC_{27}	27.35	5.52±1.76	15.53±3.64	2.70±1.02	0.87±0.69	196, 224, 379, 394 (M^+)	108.11***	1.77	40.13***
45	7-MeC_{27}	27.43	nd	nd	nd	0.06±0.09	112, 308, 379	1.74	2.66	2.85
46	5-MeC_{27}	27.53	nd	nd	nd	0.09±0.10	84, 336, 379	4.08	5.39*	5.66*
47	3-MeC_{27}	27.75	0.82±0.53	1.01±0.19	0.62±0.18	1.04±1.52	364, 379, 394 (M^+)	7.41*	0.32	1.28
48	n-C_{28}	28.00	0.23±0.08	0.71±0.82	0.45±0.22	1.03±1.56	394 (M^+)	0.20	1.30	3.36
49	14-MeC_{28}	28.23	nd	1.08±0.34	0.20±0.11	nd	210, 224, 393, 408 (M^+)	3.43	1.66	82.63***
50	8-MeC_{28}	28.32	0.31±0.17	nd	nd	nd	126, 308, 408 (M^+)	42.87***	36.62***	33.75***
51	C_{29}diene1	28.40	0.73±1.01	nd	nd	nd	404 (M^+)	39.44***	33.74***	31.13***
52	C_{29}diene2	28.51	0.28±0.15	nd	nd	nd	404 (M^+)	42.83***	36.51***	33.61***
53	4-MeC_{28}	28.60	1.52±0.50	2.09±1.46	0.63±0.32	0.54±0.58	364, 393, 408 (M^+)	0.51	0.71	2.67
54	C_{29}diene	28.67	nd	nd	nd	0.53±0.21	404 (M^+)	120.96***	140.34***	144.1***
55	(*Z*)-9-$C_{29:1}$	28.72	3.55±2.43	5.00±1.87	1.84±0.78	0.60±0.13	406 (M^+)	56.67***	2.22	23.52***
56	(*Z*)-7-$C_{29:1}$	28.79	2.69±1.39	5.29±1.98	1.39±0.21	0.64±0.27	406 (M^+)	59.75***	0.85	21.26***
57	n-C_{29}	29.00	1.50±0.35	2.54±0.42	1.53±0.33	2.72±3.47	408 (M^+)	7.46*	0.04	2.93
58	15-MeC_{29} 13-MeC_{29}	29.28	3.26±2.67	6.34±1.22	1.64±0.64	0.46±0.29	224, 407, 422 (M^+) 196, 252, 407, 422 (M^+)	117.95***	2.84	57.69***
59	7-MeC_{29}	29.42	nd	0.31±0.26	nd	nd	112, 336, 407	132.26***	105.08***	112.67***
60	3-MeC_{29}	29.73	nd	0.24±0.10	0.12±0.03	0.24±0.55	392, 407	0.07	0.90	50.26***
61	n-C_{30}	30.00	nd	nd	0.12±0.14	0.58±0.86	422 (M^+)	13.47**	0.18	0.22
62	C_{31}diene	30.49	0.65±0.09	nd	0.37±0.21	0.39±0.29	432 (M^+)	14.23**	22.60***	9.08**
63	(*Z*)-9-$C_{31:1}$	30.62	0.35±0.16	0.98±0.53	0.21±0.03	0.42±0.47	434 (M^+)	0.96	0.06	3.42
64	C_{31}monoene	30.71	1.17±0.72	nd	1.09±0.37	0.33±0.39	434 (M^+)	6.29*	24.43***	2.24
65	n-C_{31}	31.00	nd	0.48±0.22	0.20±0.07	0.75±1.16	436 (M^+)	63.71***	98.81***	107.01***
66	15-MeC_{31} 13-MeC_{31}	31.25	0.67±1.15	0.36±0.29	0.15±0.09	0.18±0.15	224, 252, 435 196, 280	0.90	0.00	2.85
67	n-C_{32}	32.00	nd	nd	nd	0.21±0.32	450 (M^+)	2.69	3.63	3.81
68	C_{33}diene	32.51	nd	nd	nd	0.13±0.09	460 (M^+)	12.39**	15.19**	15.75**
69	C_{33}monoene	32.77	nd	nd	0.47±0.17	0.12±0.18	462 (M^+)	40.68***	4.18	3.96
70	n-C_{33}	33.00	nd	nd	nd	0.10±0.23	464 (M^+)	0.19	0.54	0.63

[a] Peaks number correspond to those in Fig. 1.
[b] Percent of total hydrocarbons area; mean±SD; nd, not detected.
[c] ANOVA of Z-transformed hydrocarbon abundances. Significance levels for *F*-values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

Upon touching the female body, more than 90% of males of both species immediately mounted and held tightly, then tapped the female antennae with thier antennae and licked the female pronotum with their maxillary palpus. In addition, while tapping and licking, the males bent their abdomen to copulate. Abdominal bending was observed for all mounting males. The male orientation towards the female appeared to occur in response to movement by the female, but mate recognition did not necessarily require female motion, because the males while walking around were able to detect dead females and showed mating behavior towards them. No male-male interference affecting mounting behavior was observed.

Role of cuticular chemicals

A series of mating experiments revealed that males can discriminate between conspecific and heterospecific females, and that only non-polar cuticular chemicals have a central role in eliciting mating behavior (Table 1). More than 90% of males mounted a conspecific living female in both species (treatment 1), whereas males did not mount heterospecific females (treatment 2). At least 80% of males of both species also mounted an anesthetized conspecific female (treatment 3), but they did not mount the solvent-washed females in which cuticular chemicals had been stripped (treatment 4). In conspecific mating trials (treatments 1 and 3), the proportions of males mounting live and anesthetized females did not differ significantly in either species (treatment 1 vs. 3: Fisher's exact probability test, $P>0.1$). In addition, 70% of males of both species mounted a conspecific female coated with conspecific female extract after removal of cuticular chemicals (treatment 5). The males also showed mating behavior towards a heterospecific female coated with conspecific female extract after the cuticular chemicals had been stripped (treatment 6), although the proportion of mounting males differed between species: only 33% of the *P. grallatrix* males mounted, compared to 70% of the *P. takechii* males. The proportion of *P. grallatrix* males mounting heterospecific females was significantly lower than that mounting conspecific females (treatment 5 vs. 6: Fisher's exact probability test, $P=0.0092$). In this treatment, 80% of male *P. grallatrix* tried to mount the heterospecific female; however, all but 33% of the males did not maintain the mounting position and abandoned mating.

Cuticular hydrocarbon constituents

ANOVA showed that the relative abundance of each compound (*Z*-transformed) differed between species for 38 of 70 hydrocarbons, and between sexes for 30 of 70 hydrocarbons (Fig. 1a, b; Table 2).

The *P. grallatrix* extract contained a series of alkanes with 20–29 carbons in females and 20–31 carbons in males, and accounted for 25% and 24% of the total cuticular hydrocarbons (CHC) in females and males, respectively. The olefins were composed of 21–31 carbons in both sexes and accounted for 49% (female) and 33% (male) of the total CHC. The double-bond position was (*Z*)-9 or (*Z*)-7 in the monoenes. The methylalkanes were composed of 21–31 carbons in both sexes and accounted for 25% (female) and 43% (male) of the total CHC.

The *P. takechii* extract contained a series of alkanes with 20–31 carbons in females and 20–33 carbons in males, and accounted for 34% and 32% of the total CHC in females and males, respectively. The olefins were composed of 21–33 carbons in both sexes and accounted for 49% (female) and 32% (male) of the total CHC. The double-bond position was either (*Z*)-9 or (*Z*)-7 in the monoenes. The methylalkanes were composed of 20–31 carbons in both sexes and accounted for 17% (female) and 36% (male) of the total CHC.

Table 3. Parameters in the canonical discriminant analysis in four species/sex categories.

CHC	Eigenvector	
	CAN1	CAN2
(*Z*)-7-$C_{21:1}$	–3.59	–0.22
n-C_{21}	11.88	4.04
C_{23}diene	–0.80	0.97
C_{25}diene	–8.38	3.70
4-MeC_{26}	1.74	–0.75
C_{27}diene1	–4.14	–2.79
C_{27}diene2	7.96	1.88
(*Z*)-7-$C_{27:1}$	2.55	–2.11
15-MeC_{29}	7.06	0.81
Eigenvalue (%)	3792.72 (96.98%)	105.33 (2.67%)

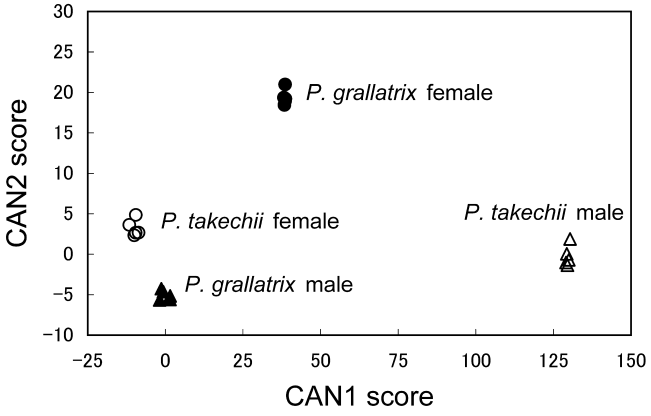

Fig. 2. Two-dimensional plot of the first and second canonical discriminant scores (CAN1, CAN2) for cuticular hydrocarbon components of male and female *P. grallatrix* and *P. takechii*. See Table 3 for the canonical discriminant function.

Stepwise discriminant analysis selected nine hydrocarbons to constitute a function discriminating four species/sex categories without error (Wilks' $\lambda<0.0001$, $F=181.20$, $P<0.0001$; Table 3): (Z)-7-$C_{21:1}$, n-C_{21}, C_{23}diene ,C_{25}diene, 4-MeC_{26}, C_{27}diene1, C_{27}diene2, (Z)-7-$C_{27:1}$, and 15-MeC_{29}. Fig. 2 shows that the combination of the first and second canonical discriminant scores (CAN1, CAN2) clearly distinguishes between species and sex.

DISCUSSION

Mating behavior

In these diurnal *Pidonia* species, a male was able to recognize a dead, motionless female as a conspecific mate and mount, after detecting the female body with its antennae. In addition, males of the two species mounted het-

erospecific females coated with conspecific extracts, despite the differences in size and coloration between females of the two species. Thus, visual detection of moving objects and discrimination by color pattern or size may not be essential in the mating behavior of *Pidonia*.

Role of cuticular chemicals in mating

Male mounting behavior was not observed towards heterospecific females, indicating that the mate-recognition cue is species-specific. In both species, males mounted anesthetized and extract-treated females but did not mount solvent-washed females. These results suggest that female cuticular chemicals play a pheromonal role and induce male mating behavior. Fukaya and Honda (1996) performed a decoy experiment on another longicorn beetle, *Psacothea hilaris*, to demonstrate that contact pheromones alone elicited male mating behavior. At least in *P. takechii*, we confirmed that a decoy, a piece of brass nail coated with a hexane extract from females, elicited male mounting behavior (50% of 10 trials) (T. Tanigaki *et al*., unpublished data). Thus, female cuticular chemicals alone can elicit male mating behavior in *Pidonia*. In the field, both species were observed on the same flowers during the same season, but heterospecific mounting was never observed. Our laboratory and field studies suggest that cuticular chemicals play an important role in the premating isolation of these sympatric *Pidonia* species.

Males of *P. grallatrix* achieved a low (33%) mounting rate against solvent-washed, conspecific extract-coated *P. takechii* female despite their active mounting attempts. This result may be explained by the mismatch in body size between sexes of the two species. Although males of both species are similar in size, females of *P. takechii* are approximately 20% smaller than those of *P. grallatrix*. It seemed that male *P. grallatrix* could not hold female *P. takechii* tightly and abandoned mounting. Thus, differences in body size may also be an important factor in premating isolation among closely related longicorn beetle species. Fukaya and Honda (1996) also showed that body size is an important factor for the success of mounting and copulation, although contact pheromones alone invoked male mating behavior.

Cuticular hydrocarbon constituents

GC–MS analysis revealed that the cuticular chemicals were composed of several types of hydrocarbons. Although approximately 70 different chemicals were distinguished, extracts of previously examined insects contained 100 or more chemicals (Howard, 1993). The major components of the extracts analyzed in this study were olefins, *n*-alkanes, and methylalkanes. In particular, the predominance of olefins in the cuticular lipids differs from previous findings in insects (Lockey, 1988), in which *n*-alkanes predominated.

The hydrocarbon components of the two *Pidonia* species showed sexual and species differences. These differences may provide these two sympatric species with prezygotic isolation, as is the case in *Drosophila* (Higgie *et al*., 2000; Howard *et al*., 2003). The compounds selected in the stepwise discriminant analysis included (*Z*)-7-$C_{27:1}$, which is one of the previously reported sex pheromones of the longicorn beetle *Anoplophora glabripennis* (Zhang *et al*., 2003). (*Z*)-9-$C_{23:1}$ and (*Z*)-9-$C_{25:1}$ showed significant sexual differences (Table 2), although these were not selected in the stepwise discriminant analysis. These are also known as sex pheromones in some longicorn beetles (Zhang *et al*., 2003; Ginzel *et al*., 2003a). Thus, cuticular hydrocarbons that showed sexual and/or species difference may include sex pheromones. However, we have not studied which components are responsible for the recognition of species and sex by the beetles. In future studies, we need to identify the pheromonal component of female cuticular hydrocarbons and determine whether a single cuticular hydrocarbon component or a combination of some components acts as a pheromone. In addition, analysis of geographical variation in cuticular hydrocarbons is needed to understand their role in prezygotic isolation in different assemblages of *Pidonia* species.

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